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Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8+ T-cell responses

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CD8+ T cells are essential for long-term, vaccine-induced resistance against intracellular pathogens. Here we show that natural antibodies, acting in concert with complement, are endogenous adjuvants for the generation of protective CD8+ T cells after vaccination against visceral leishmaniasis. IL-4 was crucial for the priming of vaccine-specific CD8+ T cells, and we defined the primary source of IL-4 as a CD11b+CD11c^{lo} phagocyte. IL-4 secretion was not observed in antibody-deficient mice and could be reconstituted with serum from normal, but not *Btk* immune-deficient, mice. Similarly, no IL-4 response or CD8+ T-cell priming was seen in *C1qa*$^{-/-}$ mice. These results identify a new pathway by which immune complex–mediated complement activation can regulate T-cell-mediated immunity. We propose that this function of natural antibodies could be exploited when developing new vaccines for infectious diseases.

The importance of CD8+ T cells in host protection against intracellular pathogens is well recognized[1–3], and CD8+ T-cell priming is crucial for successful vaccination against leishmaniasis[4]. Although genome sequencing projects now provide a plethora of potential vaccine candidates[5], few criteria exist for selecting from them, and antigen choice remains largely empirical. In addition, successful vaccination requires antigen targeting to the major histocompatibility class I processing pathway[6–8] and a cytokine milieu that favors CD8+ T-cell priming and differentiation[3,9]. Recent interest has focused on how antibody and complement regulate CD8+ T-cell-mediated immunity. Fc receptor (FcR)-mediated uptake of immune complexes facilitates class I–restricted antigen presentation[6,7], leading to the suggestion that vaccine efficacy might be improved by immunization with preformed immune complexes[6,7]. Mice deficient in complement component C3 (*C3*$^{-/-}$) mount poor cytotoxic T-lymphocyte (CTL) responses to infection with influenza virus[10] and lymphocytic choriomeningitis virus[11]. However, neither complement receptor (CR)1 nor CR2 seem crucial to the regulation of CD8+ T cells, in contrast to their roles in promoting humoral and CD4+ T-cell responses[12]. Precisely how complement regulates CD8+ T-cell responses remains unknown.

Natural antibodies are the germline-encoded IgM, IgG and IgA found in normal humans and other animals[13,14]. They have low affinity compared with hypermutated antibodies generated during normal immune responses, and have broad reactivity to a variety of self-antigens[14]. Most natural antibodies are produced by self-renewing B-1 B cells, which preferentially localize to the peritoneum[13]. Natural antibodies serve as innate microbial recognition receptors, recognizing various bacterial cell wall components[15]. Natural antibodies to phosphorylcholine can protect against pneumococcal disease[15], and natural antibody-dependent complement activation has a role in lipopolysaccharide clearance and protection from endotoxemia[16]. A specific role for natural antibodies in the regulation of acquired T-cell-mediated immunity during infection or vaccination has not been described.

We now show a direct and previously unrecognized link between recognition of pathogen-derived molecules by natural antibodies and induction of a new complement-dependent, IL-4-mediated pathway of CD8+ T-cell priming. Our study brings together a number of independent observations on the role of antibody and complement in CD8+ T-cell activation. These data form the basis of a coherent model for how immune complexes involving natural antibodies act as a bridge between innate and vaccine-induced acquired immunity. In addition, we suggest a new approach for the selection of candidate vaccine antigens, emphasizing their recognition by natural antibodies.

RESULTS

IL-4-dependent priming of CD8+ T cells

Hydrophilic acylated surface protein (HASP)B-1, a member of a family of proteins expressed by infective stages of *Leishmania* parasites[17], was recently identified by us as a lead candidate for vaccination

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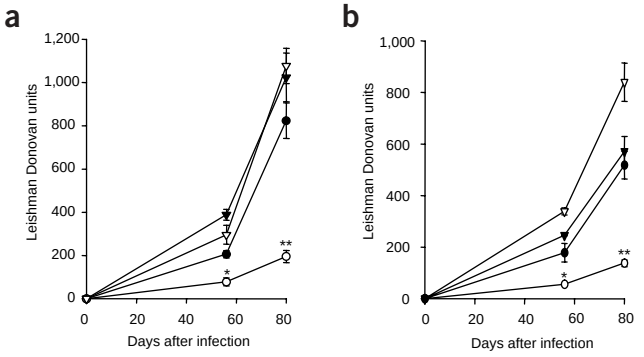


Figure 1 IL-4 is required for vaccination against leishmaniasis. (**a**) Groups of control (●, ▲) and vaccinated (○, △) BALB/c (●, ○) and *Il4*$^{-/-}$ (▲, △) mice ($n = 5$ per group) were challenged with *L. donovani*, and parasite burden (expressed in Leishman Donovan units[2]) in the spleen was determined at the times indicated. (**b**) Parasite burden of BALB/c mice (●, ○) compared with *Il4ra*$^{-/-}$ mice (▲, △), as in **a**. *, $P < 0.05$; **, $P < 0.01$ compared with unvaccinated mice.

against visceral leishmaniasis[2]. Immunization with recombinant HASPB-1 induced antigen-specific CD8$^+$ T cells and long-term protection, which are both characteristics attributed to DNA vaccines. However, neither protective vaccination nor generation of CD8$^+$ T cells required the use of exogenous adjuvant or artificial delivery systems[2]. As part of a study to determine the cytokine requirements for protection in this model, we immunized IL-4-deficient mice with recombinant HASPB-1 and challenged them with *Leishmania donovani*. Compared with control BALB/c mice, *Il4*$^{-/-}$ mice were not protected (**Fig. 1a**). Vaccination also failed in *Il4ra*$^{-/-}$ mice, which lack responsiveness to both IL-4 and IL-13 (**Fig. 1b**). To assess whether this result was obtained because recombinant HASPB-1-specific CD8$^+$ T cells were not primed, we isolated spleen cells from vaccinated animals before challenge, restimulated them with recombinant HASPB-1 and assessed the frequency of IFN-γ-producing cells. The frequency of HASPB-1-specific CD8$^+$ T cells increased after vaccination of BALB/c (**Fig. 2a**), but not *Il4*$^{-/-}$, mice (**Fig. 2b**). As an alternate measure of CD8$^+$ T-cell priming, we assayed HASPB-1-specific CTL activity. CTLs that were able to kill HASPB-1-expressing target cells were readily expanded from vaccinated BALB/c, but not *Il4*$^{-/-}$, mice (**Fig. 2c**). Finally, we examined the protective capacity of these CD8$^+$ T cells using an adoptive transfer approach. CD8$^+$ T cells from vaccinated BALB/c, but not *Il4*$^{-/-}$, mice could transfer protection to both the spleen (**Fig. 2d**) and liver (**Fig. 2e**) of infected BALB/c recipients. Together, these experiments indicated for the first time a crucial requirement for IL-4 in CD8$^+$ T-cell-dependent vaccination against leishmaniasis.

CD11b$^+$CD11c^{lo} phagocytes provide early IL-4

Recombinant HASPB-1 induced rapid IL-12p40 and IL-12p70 production in CD11c^{hi} dendritic cells[2]. We used BALB/c and *Il4ra*$^{-/-}$ mice to determine whether injection of recombinant HASPB-1 would result in secretion of both IL-4 and IL-12. As *Il4ra*$^{-/-}$ mice do not respond to IL-4, they provided a means of dissecting any temporal relationships between IL-4 and IL-12 secretion. An increase in the frequency of IL-4-secreting cells was observed in both strains injected with recombinant HASPB-1 (**Fig. 3a**). In contrast, BALB/c mice had an increased frequency of IL-12p70-secreting cells, whereas *Il4ra*$^{-/-}$ mice did not (**Fig. 3b**). These data placed IL-4 upstream of IL-12p70 production, supporting an instructional role for IL-4 (ref. 18). IL-12 produced in response to IL-4 must also be involved in providing an appropriate cytokine milieu for CD8$^+$ T-cell priming, as *Il12*$^{-/-}$ mice were also unable to prime HASPB-1-specific CD8$^+$ T cells after vaccination (**Fig. 3c**).

In BALB/c (but not *Il4ra*$^{-/-}$) mice, IL-4 secretion was observed in populations enriched for CD11c^{hi} cells (~70–80% conventional dendritic cells) and CD11c$^-$CD4$^+$ cells (~80% CD4$^+$ T cells). This result indicated that conventional CD11c^{hi} dendritic cells and CD4$^+$ T cells only secrete IL-4 in response to IL-4Rα signaling. The primary source of IL-4 in *IL4ra*$^{-/-}$ mice, identified by magnetic bead–activated cell sorting (MACS), was CD11c$^{lo/neg}$CD4$^-$cells (**Fig. 4a**), which could be positively selected using monoclonal antibody to CD11b (**Fig. 4b**). To further characterize the IL-4 response in these CD11b$^+$ cells, we used real-time RT-PCR to analyze the accumulation of IL-4 mRNA. MACS-enriched (~80%) CD11b$^+$ cells, but not CD11b-depleted spleen cells (<1% CD11b$^+$), showed a rapid and transient IL-4 response after administration of recombinant HASPB-1 (**Fig. 4c**). To confirm the identity of these CD11b$^+$ cells, we used IL-4–enhanced green fluorescent protein (EGFP) reporter mice ('4get' mice)[19]. We identified a distinct population of EGFP$^+$CD11b$^+$CD11c^{lo} cells (~0.5% of total spleen cells) in mice that had received recombinant HASPB-1, but not in control untreated mice (**Fig. 4d**). To examine whether these cells belonged to the mononuclear phagocyte lineage, we administered liposomes containing clodronate (dichloromethyl-

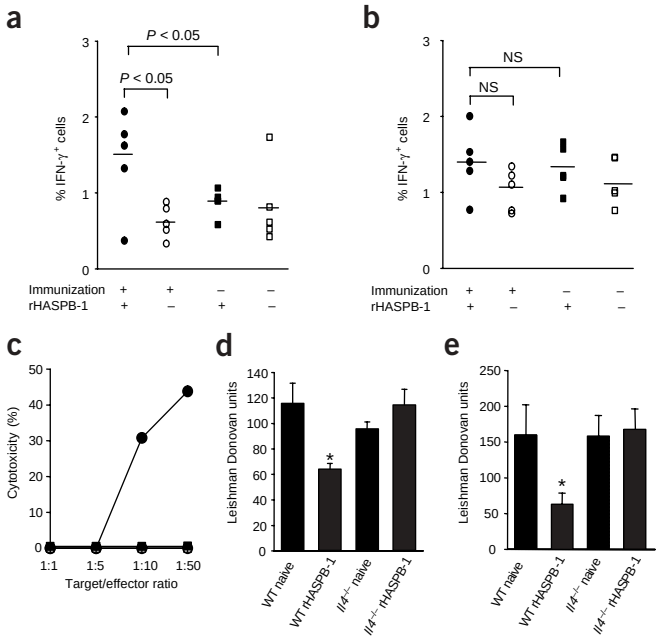


Figure 2 Priming of recombinant HASPB1–specific CD8$^+$ T cells requires IL-4. (**a**,**b**) Spleen cells from individual BALB/c mice (**a**) and *Il4*$^{-/-}$ mice (**b**) were immunized with recombinant HASPB-1 (rHASBP-1; ●, ○) or ovalbumin (■, □), cultured with (●, ■) or without (○, □) recombinant HASPB-1 and assayed for IFN-γ-producing CD8$^+$ T cells. NS, not significant. Horizontal bars indicate mean values. (**c**) CTL activity of BALB/c-derived (●, ○) and *Il4*$^{-/-}$-derived (■, □) CD8$^+$ T cells against HASPB-1-expressing (●, ■) or control (○, □) targets. (**d**,**e**) CD8$^+$ T cells from naive or vaccinated BALB/c or *Il4*$^{-/-}$ mice were transferred into BALB/c recipients, and parasite burden in spleen (**d**) and liver (**e**) was determined on day 50 after infection. WT, wild-type. *, $P < 0.05$ compared with mice receiving cells from unvaccinated mice.

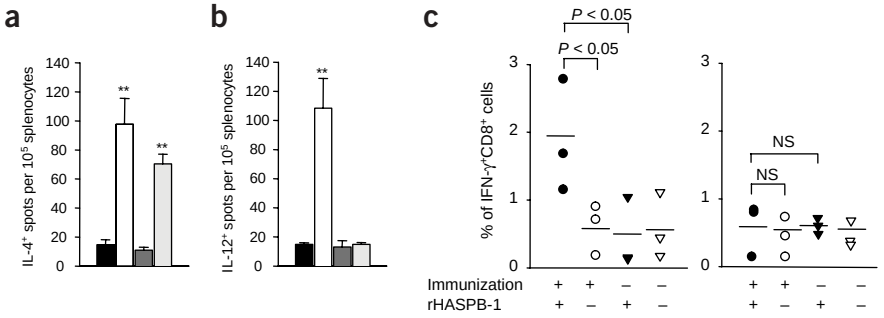
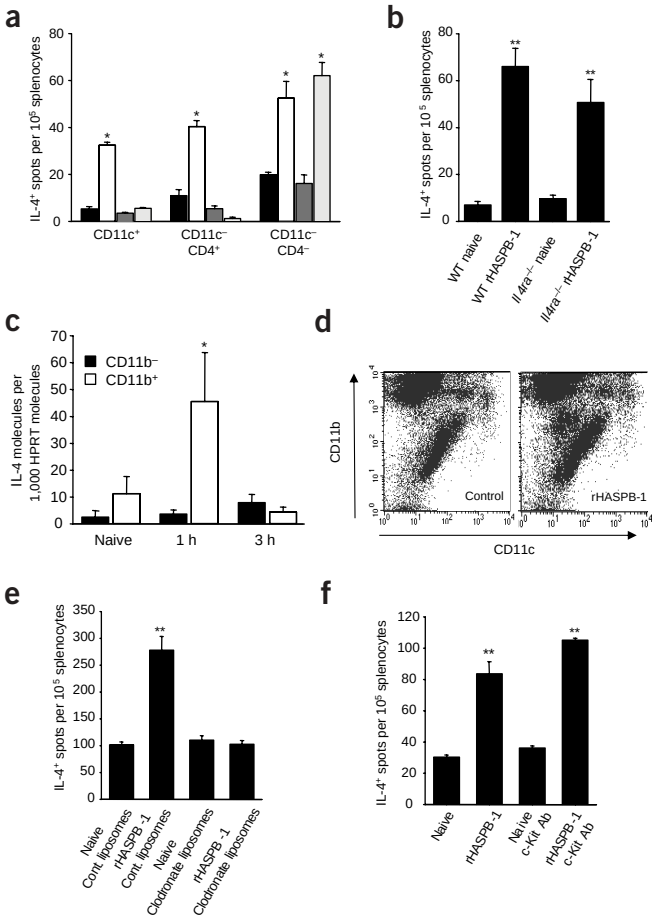


Figure 3 IL-4 secretion is upstream of IL-12 production. (**a**,**b**) IL-4 (**a**) and IL-12 (**b**) responses of spleen cells from BALB/c (■, □) and *Il4ra*$^{-/-}$ (■, ■) mice that were untreated (■, ■) or injected with recombinant HASPB-1 (rHASPB-1; □, ■). **, $P < 0.01$ compared with untreated mice. (**c**) Spleen cells from individual C57BL/6 (left) and *Il12*$^{-/-}$ (right) mice were immunized with recombinant HASPB-1 (●, ○) or ovalbumin (▲, △) were cultured with (●, ▲) or without (○, △) recombinant HASPB-1 and assayed for IFN-γ-producing CD8$^+$ T cells. NS, not significant.

ene bisphosphonate). This treatment, which selectively induces apoptosis in mononuclear phagocytes (including monocytes, macrophages and dendritic cells)[20], completely ablated IL-4 secretion (**Fig. 4e**). Mast cells and basophils have been reported to express IL-4 (refs. 21,22), but we observed no increase in the frequency of EGFP$^+$Fc-εR^{hi} cells in 4get mice treated with recombinant HASPB-1 (data not shown). In addition, *in vivo* depletion of mast cells with monoclonal antibody to c-Kit did not alter the response to recombinant HASPB-1 (**Fig. 4f**). Similarly, *in vivo* depletion of granulocytes and plasmacytoid dendritic cells with Gr1 monoclonal antibody and depletion of natural killer cells with ASGM-1 antiserum had no effect on IL-4 secretion (data not shown). Together, these data indicate that CD11b$^+$CD11c^{lo} mononuclear phagocytes are the primary source of IL-4 secretion after injection of recombinant HASPB-1.



Production of IL-4 requires natural antibodies

We injected recombinant HASPB-1 into severe combined immunodeficiency (SCID) mice and, unexpectedly, observed no IL-4 response. However, after transferring normal serum from BALB/c mice to the SCID mice, IL-4 secretion in response to recombinant HASPB-1 was restored (**Fig. 5a**). This experiment further excluded the possibility that T cells, B cells and CD4$^+$ natural killer T cells act as a primary source of IL-4, and indicated a role for serum antibodies. IL-4 secretion was also absent in B-cell-deficient mice, but was restored after serum transfer (data not shown).

Normal serum contains a substantial amount of natural IgM antibodies, secreted predominantly by B-1a cells. To determine whether such antibodies might be involved in the IL-4 response to HASPB-1, we took two approaches. First, we sorted B220$^+$CD5$^+$IgD^{dull} peritoneal B-1a cells and showed that they spontaneously produced HASPB-1-specific IgM *in vitro* (**Fig. 5b**). At the level of cell plating in these assays, we did not detect IgG3 secretion by these cells. Peritoneal B220$^+$IgM^{pos}IgD^{dull} B cells, including both B-1a and B-1b cells[23,24], also spontaneously secreted IgM that recognized HASPB-1, though at lower frequency than in sorted B-1a cells (data not shown). Next, we tested the role of B-1 cell–derived antibodies in the IL-4 response, using adoptive transfer of serum from CBA/N mice. CBA/N mice carry the *Btk* mutation[25] and are severely deficient in B-1 cells, as well as having reduced numbers of conventional B cells[26]. Serum from wild-type CBA/J, but not mutant CBA/N, mice restored the capacity of SCID mice to secrete IL-4 (**Fig. 5c**). Collectively, these data strongly suggest that B-1 B-cell-derived IgM natural antibodies initiate the primary IL-4 response to recombinant HASPB-1. We also found that serum from IgM-deficient mice[27] was able to transfer the IL-4-secreting capacity to SCID mice (**Fig. 5d**), suggesting that, at least in IgM-deficient mice, other natural antibody isotypes may accumulate in serum to a biologically significant level. To extend our observations on immune complex–mediated IL-4 secretion, we transferred normal or ovalbumin-specific immune serum into SCID mice before injecting

Figure 4 CD11b$^+$CD11c^{lo} phagocytes are responsible for primary IL-4 secretion. (***a***) IL-4 secretion by MACS-separated spleen cells from control BALB/c (■) and *IL4ra*$^{-/-}$ (■) mice, and BALB/c (□) and *Il4ra*$^{-/-}$ (■) mice injected with recombinant HASPB-1 (rHASPB-1). (**b**) IL-4 secretion by CD11b$^+$ cells from control (■) or HASPB-1-injected (□) mice. (**c**) IL-4 mRNA accumulation in CD11b$^+$ (□) and CD11b$^-$ (■) cells after injection of recombinant HASPB-1 ($n = 4$–5 mice per time point). (**d**) EGFP$^+$ spleen cells from control 4get mice (left) and spleen cells 3 h after injection of recombinant HASPB-1 (right). Cells were gated on FL-1 and then analyzed for CD11b and CD11c. Control spleen cells contain abundant (~6–7% of total spleen cells) autofluorescent and spontaneously EGFP$^+$ cells. (**e**,**f**) IL-4 secretion by spleen cells from naive or HASPB-1-injected BALB/c mice, treated with clodronate liposomes (**e**) or c-Kit monoclonal antibody (**f**). *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice.

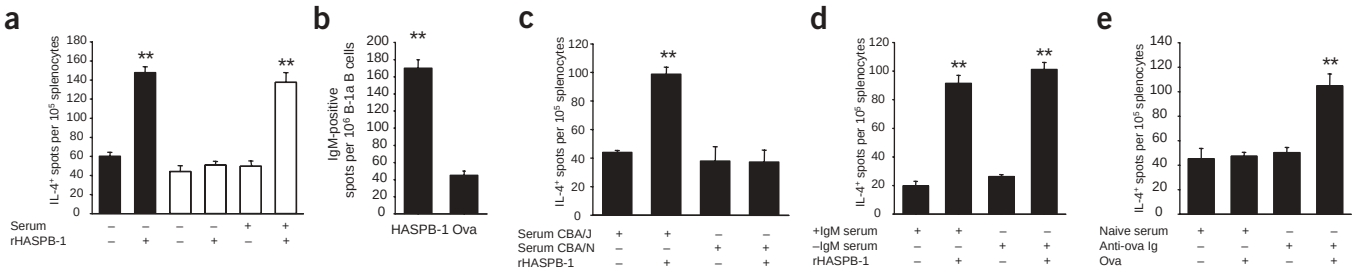


Figure 5 IL-4 secretion requires immune complexes. (**a**) IL-4 responses of spleen cells from BALB/c (■) or SCID (□) mice injected with recombinant HASPB-1 (rHASPB-1), with or without serum transfer. (**b**) Frequency of FACS-purified B220$^+$CD5$^+$IgD^{dull} peritoneal B-1a cells secreting IgM antibodies that recognize HASPB-1 or ovalbumin (Ova). (**c**) IL-4 response to recombinant HASPB-1 in SCID mice reconstituted with serum from wild-type CBA/J or mutant CBA/N mice. (**d**) IL-4 responses of SCID mice reconstituted with serum from wild-type (+IgM) or IgM-deficient (–IgM) BALB/c mice. (**e**) IL-4 responses in SCID mice injected with ovalbumin in the presence or absence of antibodies to ovalbumin (Anti-ova Ig). *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice (**a**,**c**,**d**) or the IgM response to ovalbumin (**b**).

them with ovalbumin. In the presence of specific antibodies to ovalbumin, ovalbumin injection induced IL-4 secretion comparable to that induced by recombinant HASPB-1 (**Fig. 5e**). We therefore concluded that immune complexes can, in general, provide a means of stimulating primary IL-4 secretion from CD11b$^+$CD11c^{lo} phagocytes.

Complement activation induces IL-4 secretion

One consequence of immune complex formation is activation of the classical complement pathway. We used two approaches to evaluate whether complement activation had any role in regulating the IL-4 response described above. After depletion of complement with cobra venom factor, both BALB/c (**Fig. 6a**) and serum-transferred SCID (data not shown) mice were unable to secrete IL-4 in response to recombinant HASPB-1. In addition, the frequency of HASPB-1-induced EGFP$^+$CD11b$^+$CD11c^{lo} cells (**Fig. 4d**) cells was reduced by 82% in 4get mice treated with cobra venom factor (data not shown). Confirming the downstream consequences of IL-4 deficiency, complement-depleted BALB/c mice were also unable to mount a detectable IL-12 response (**Fig. 6b**). C1q-deficient mice[28] were used to confirm the importance of classical pathway activation. Neither IL-4 (**Fig. 6c**) nor IL-12 (**Fig. 6d**) secretion was observed in *C1qa*$^{-/-}$ mice. Furthermore, these mice could not prime recombinant HASPB-1-specific CD8$^+$ T cells (**Fig. 6e,f**). Together, our results define immune complex–mediated complement activation as a crucial step in the generation of IL-4-dependent CD8$^+$ T-cell responses after vaccination with recombinant HASPB-1.

DISCUSSION

Our results show that the innate antibody repertoire, in conjunction with complement, can provide an instructional role for the generation of CD8$^+$ T-cell responses. The first important observation underpinning this conclusion is the identification of IL-4 as a crucial cytokine for priming HASPB-1-specific CD8$^+$ T cells and for vaccine-induced protection against visceral leishmaniasis. IL-4 has a significant role in the induction of tumor-specific and some, but not all, antiviral CD8$^+$ T-cell responses[29,30]. An essential requirement for IL-4 in vaccine-induced CD8$^+$ T-cell responses has not been previously described, and might explain why *Leishmania* vaccines using strong IFN-γ-promoting adjuvants have met with only partial success compared with vaccines containing alum[31,32]. Our data and that of others[18,33,34] place IL-4 at the head of a cytokine cascade involved either directly or indirectly in instructing T-cell priming and differentiation. Nevertheless, identifying the primary source(s) of IL-4 has been extremely difficult and has led to controversial results[21,35]. Our second major observation, therefore, is that CD11b$^+$CD11c^{lo} clodronate-sensitive phagocytes are the primary source of IL-4 in this system. Recently, a CD11b$^+$CD11c^{int} pre-dendritic cell population has been identified in mouse peripheral blood[36]. On the basis of our phenotyping to date, the pre-dendritic cell is a possible candidate for the IL-4-secreting cell described here. However, we cannot rule out other subpopulations of monocytes or macrophages, which may have low levels of CD11c expression. Although dendritic cells and other mononuclear phagocytes are not often associated with IL-4 secretion, several previous reports indicate that they have this potential[37,38] and

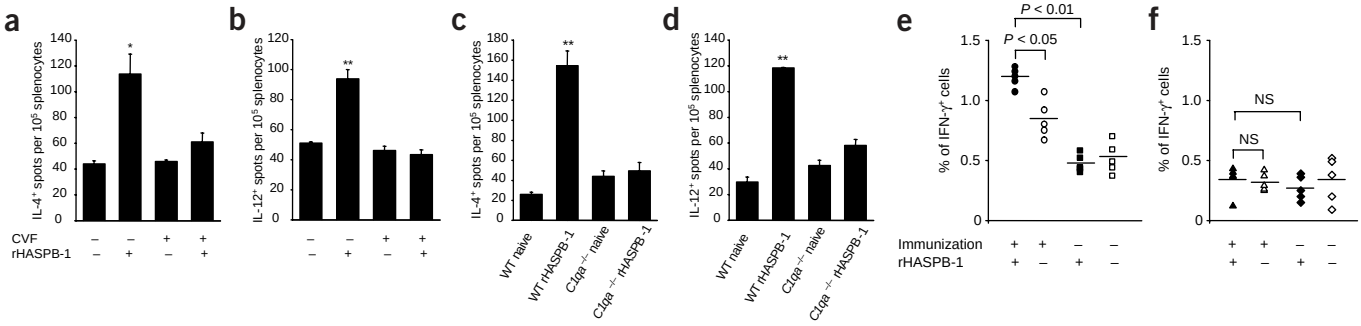


Figure 6 Cytokine secretion and CD8$^+$ T-cell priming are complement-dependent. (**a**,**b**) Secretion of IL-4 (**a**) and IL-12p70 (**b**) in BALB/c mice depleted of complement with cobra venom factor (CVF). (**c**,**d**) IL-4 (**c**) and IL-12p70 (**d**) secretion in B6 and *C1qa*$^{-/-}$ mice in response to recombinant HASPB-1 (rHASPB-1). (**e**,**f**) CD8$^+$ T-cell responses in B6 (**e**) and *C1qa*$^{-/-}$ (**f**) mice immunized with recombinant HASPB-1 (●, ○, ▲, △) or ovalbumin (■, □, ◆, ◇), with or without restimulation with recombinant HASPB-1. *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice; NS, not significant.

this lineage has considerably more functional heterogeneity *in vivo* than its often-studied bone marrow–derived counterparts. Further studies with an extended panel of cell surface markers are clearly warranted to obtain an unambiguous identification. Nevertheless, the identification of a pathway of IL-4 secretion linked to complement activation may have important implications for understanding the role of complement in various aspects of acquired immunity. IL-4 has a major role, both directly and indirectly, in $CD4^+$ T-cell differentiation[33], B-cell activation[39] and regulation of macrophage antimicrobial function[40]. Our study suggests that the potential involvement of IL-4 in some previously reported complement-dependent effects[41,42] must now be carefully addressed.

The consequences of pathogen recognition by natural antibodies have usually been thought of simply in terms of opsonization and clearance[15,16]. The third major conclusion from our study, therefore, is that natural antibody recognition of pathogen-derived proteins can act as a trigger for complement-dependent pathway(s) leading to IL-4 production. Hence, this property of natural antibodies endows them with natural adjuvant activity. The failure of serum from *Btk* mice to transfer IL-4-secreting capacity, as well as the production of HASPB-1-specific IgM antibodies by peritoneal B-1a B cells, strongly suggests that natural antibodies recognize this *Leishmania* protein and underpin its efficacy as a vaccine. The structural features of HASPB-1 that impart recognition by natural antibodies are presently unknown, but it is notable that *L. donovani* HASPB-1 (together with other members of the HASPB-1 family) contains multiple repeats[17], a feature shared with other targets of natural antibody recognition[43].

The availability of full genome sequence data for many important human pathogens presents major challenges for the screening and identification of new vaccine candidate antigens[5]. Our observation that immune complexes formed with natural antibodies can result in IL-4 secretion and efficient $CD8^+$ T-cell priming leads us to suggest that natural antibodies could be used to identify potential vaccine candidate antigens. In cases such as this study, where IL-4-dependent responses are clearly essential for vaccine efficacy, the use of natural antibodies may provide a convenient means of selecting from a vast array of potential vaccine candidates. In contrast, where IL-4-dependent responses may be detrimental, it may be preferable to choose antigens that are not targets for natural antibody recognition.

METHODS

Mice and parasites. We used the following mouse strains (kept under barrier conditions): BALB/c (Tuck); CBA/J and C57BL/6 (B6; Charles River); BALB/c-background *Il4*$^{-/-}$ and *Il4ra*$^{-/-}$ (ref. 44), IgM-deficient (ref. 27) and SCID (National Institiute for Medical Research); and B6-background *C1qa*$^{-/-}$ (ref. 28), *Il12*$^{-/-}$ (American Type Culture Collection), 4get (ref. 19; ATCC) and B-cell-deficient (B & K Universal). *L. donovani* amastigotes (strain LV9) were isolated from infected hamsters as described[2]. Mice were infected with 2×10^7 amastigotes through the lateral tail vein. Hepatic and splenic parasite burdens were determined from Giemsa-stained tissue impression smears, and data were presented in Leishman Donovan units (LDU)[2]. All animal procedures were approved by the institutional Animal Procedures Ethics Committees and the UK Home Office.

Antigen preparation and vaccination. Recombinant HASPB-1 was either purified as described[2] or by reverse-phase high-performance liquid chromatography (97% purity; 0.17 endotoxin units per mg recombinant HASPB-1; Dictagene). Mice were vaccinated subcutaneously with recombinant HASPB-1 or ovalbumin (Sigma-Aldrich) and infected 3 weeks later.

Flow cytometry. Spleen cells from vaccinated mice were cultured overnight with or without recombinant HASPB-1 and recombinant IL-2, and stained with Tricolor-labeled antibody to CD8 (clone CT-CD8α; Caltag) and R-phycoerythrin-labeled antibody to IFN-γ (clone BVD6-24G2; Serotec)[2]. Isotype controls were used to set markers. Samples were analyzed using a FACSCaliber (Becton Dickinson) and CellQuest software. To identify IL-4-producing cells in 4get mice, an FL-1 gate was set to include all potential $EGFP^+$ and autofluorescent cells. Gated cells were subsequently analyzed for CD11b and CD11c expression. Fc-εR^+ cells were identified by staining with purified mouse IgE, biotinylated antibody to mouse IgE and streptavidin-allophycocyanin (all from PharMingen)[21,45].

CTL assay. P815 cells were stably transfected with the pCIneo vector, bearing the open reading frame of HASPB-1 (ref. 17), or the vector alone, and used as targets. Effector $CD8^+$ T cells were expanded from vaccinated mice with 30 μg/ml recombinant HASPB-1. Recombinant IL-2 (20 U/ml) was added 3 d later, and $CD8^+$ cells were purified at day 7 by MACS using CD8 antibodies bound to microbeads (Miltenyi Biotec). CTL activity was measured in a 4-h lactate dehydrogenase release assay (Boehringer Mannheim). Data were expressed as percentage of specific lysis, defined as (experimental lysis – spontaneous lysis) ÷ (maximum lysis – spontaneous lysis) × 100. Total spontaneous release was calculated as the sum of spontaneous release by target and effector cells.

Adoptive transfer of $CD8^+$ cells. $CD8^+$ T cells from vaccinated mice were isolated by MACS (1–2.5% $CD4^+$ cell contamination). Each animal received $3–4 \times 10^6$ $CD8^+$ cells intravenously. Recipient mice were infected 1 d after transfer, and parasite burden was assessed.

ELISpot assays. Splenocytes or MACS-purified subpopulations were directly assayed *ex vivo* 5 h after injection by ELISpot for IL-4 and IL-12p70 production as described[2,46]. The number of spots per 10^5 total spleen cells was calculated from triplicate wells for each mouse ($n = 3$). After separation, cell suspensions were adjusted and plated to represent the contribution each population makes to the response of 10^5 unfractionated spleen cells.

We used an ELISpot assay[16] with alkaline phosphatase–conjugated antibody to mouse IgM (μ-chain specific) and horseradish peroxidase–conjugated antibody to mouse IgG3 (Serotec) to determine the frequency of cells secreting antibody to HASPB-1. Ovalbumin was used as a control protein. $B220^+CD5^+IgD^{dull}$ B-1a cells and $B220^{dull/pos}IgM^{pos}IgD^{dull}$ B-1a and B-1b cells[23] were identified in nonadherent peritoneal cells after staining with FITC-conjugated antibody to IgD (clone 11-26c.2a), biotinylated antibody to IgM (clone II/41), phycoerythrin-conjugated antibody to B220 (RA3-6B2), peridinin chlorophyll protein–conjugated antibody to CD5 (53-7.3; all from PharMingen) or streptavidin-allophycocyanin as required, and sorted to >98% purity using a FACSVantage (Becton Dickinson).

Serum transfer and depletions. 500 μl of normal or CBA/N serum was administered intraperitoneally into SCID or μMT mice 1 d before intravenous injection of recombinant HASPB-1. $Gr1^+$ cells were depleted (by >95% for neutrophils and >85% for plasmacytoid dendritic cells) using 200 μg RB6-8C5 (ref. 47). Natural killer cells were depleted using polyclonal antibody to asialoGM1 (Cedarlane Laboratories). For mononuclear phagocyte depletion, mice received 200 μl of dichloromethylene bisphosphonate[48] or control liposomes intravenously, 1 d before recombinant HASPB-1. Mast cells were depleted with 1 mg antibody to c-Kit (ACK2; ref. 49), given intraperitoneally 1 d before recombinant HASPB-1 injection. Depletions were confirmed by histology. Complement was depleted with 30 μg cobra venom factor (Quidel) intravenously, 18 h and again 3 h before recombinant HASPB-1 injection.

Real-time RT-PCR. Real-time RT-PCR was carried out as previously described[50]. RNA was isolated from spleen tissue using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and reverse-transcribed into cDNA. We used the following oligonucleotides: *Il4* forward, 5′-CCTCACAGCAACGAAGAACA-3′; *Il4* reverse, 5′-TGGACTCATTCATGGTGCAG-3′; hypoxanthine guanine phosphoribosyl transferase (*Hprt*), as described[50]. The number of *Il4* and *Hprt* cDNA molecules in each sample was calculated using QuantiTect SYBR Green Master Mix (Qiagen) and an ABI Prism 7000 sequence detection system (Applied Biosystems). Standard curves were constructed with known amounts of *Il4* and *Hprt* cDNA.

Statistical analysis. Statistical analysis was done using a paired Student *t*-test or *U*-test depending on the sample size. $P < 0.05$ was considered significant. Each experiment was repeated at least twice, with similar results.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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1. Yoshida, A., Nagata, T., Uchijima, M. & Koide, Y. Protective CTL response is induced in the absence of CD4+ T cells and IFN-gamma by gene gun DNA vaccination with a minigene encoding a CTL epitope of *Listeria monocytogenes*. *Vaccine* **19**, 4297–4306 (2001).
2. Stager, S., Smith, D.F. & Kaye, P.M. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *J. Immunol.* **165**, 7064–7071 (2000).
3. Carvalho, L.H. *et al.* IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat. Med.* **8**, 166–170 (2002).
4. Gurunathan, S., Wu, C.Y., Freidag, B.L. & Seder, R.A. DNA vaccines: a key for inducing long-term cellular immunity. *Curr. Opin. Immunol.* **12**, 442–447 (2000).
5. Gardner, M.J. *et al.* Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**, 498–511 (2002).
6. Kalergis, A.M. & Ravetch, J.V. Inducing tumor immunity through the selective engagement of activating Fcgamma receptors on dendritic cells. *J. Exp. Med.* **195**, 1653–1659 (2002).
7. Regnault, A. *et al.* Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med.* **189**, 371–380 (1999).
8. Tobery, T.W. & Siliciano, R.F. Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the induction of *de novo* CTL responses *in vivo* after immunization. *J. Exp. Med.* **185**, 909–920 (1997).
9. Dranoff, G. *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA* **90**, 3539–3543 (1993).
10. Kopf, M., Abel, B., Gallimore, A., Carroll, M. & Bachmann, M.F. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. *Nat. Med.* **8**, 373–378 (2002).
11. Suresh, M. *et al.* Complement component 3 is required for optimal expansion of CD8 T cells during a systemic viral infection. *J. Immunol.* **170**, 788–794 (2003).
12. Haas, K.M. *et al.* Complement receptors CD21/35 link innate and protective immunity during *Streptococcus pneumoniae* infection by regulating IgG3 antibody responses. *Immunity* **17**, 713–723 (2002).
13. Hayakawa, K., Hardy, R.R. & Herzenberg, L.A. Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression. *Eur. J. Immunol.* **16**, 450–456 (1986).
14. Hardy, R.R., Carmack, C.E., Li, Y.S. & Hayakawa, K. Distinctive developmental origins and specificities of murine CD5+ B cells. *Immunol. Rev.* **137**, 91–118 (1994).
15. Martin, F., Oliver, A.M. & Kearney, J.F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**, 617–629 (2001).
16. Reid, R.R. *et al.* Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. *J. Immunol.* **159**, 970–975 (1997).
17. Alce, T.M., Gokool, S., McGhie, D., Stager, S. & Smith, D.F. Expression of hydrophilic surface proteins in infective stages of *Leishmania donovani*. *Mol. Biochem. Parasitol.* **102**, 191–196 (1999).
18. Biedermann, T. *et al.* IL-4 instructs TH1 responses and resistance to *Leishmania major* in susceptible BALB/c mice. *Nat. Immunol.* **2**, 1054–1060 (2001).
19. Mohrs, M., Shinkai, K., Mohrs, K. & Locksley, R.M. Analysis of type 2 immunity *in vivo* with a bicistronic IL-4 reporter. *Immunity* **15**, 303–311 (2001).
20. Naito, M. *et al.* Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *J. Leukoc. Biol.* **59**, 133–138 (1996).
21. Seder, R.A. *et al.* Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. *Proc. Natl. Acad. Sci. USA* **88**, 2835–2839 (1991).
22. Coffman, R.L. & von der Weid, T. Multiple pathways for the initiation of T helper 2 (Th2) responses. *J. Exp. Med.* **185**, 373–375 (1997).
23. Wardemann, H., Boehm, T., Dear, N. & Carsetti, R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J. Exp. Med.* **195**, 771–780 (2002).
24. Boes, M. *et al.* Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J. Immunol.* **160**, 4776–4787 (1998).
25. Bona, C., Mond, J.J. & Paul, W.E. Synergistic genetic defect in B-lymphocyte function. I. Defective responses to B-cell stimulants and their genetic basis. *J. Exp. Med.* **151**, 224–234 (1980).
26. Khan, W.N. *et al.* Defective B cell development and function in Btk-deficient mice. *Immunity* **3**, 283–299 (1995).
27. Lutz, C. *et al.* IgD can largely substitute for loss of IgM function in B cells. *Nature* **393**, 797–801 (1998).
28. Botto, M. *et al.* Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet.* **19**, 56–59 (1998).
29. Schuler, T. *et al.* Generation of tumor-associated cytotoxic T lymphocytes requires interleukin 4 from CD8(+) T cells. *J. Exp Med.* **194**, 1767–1775 (2001).
30. King, C. *et al.* Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T-cell responses. *Nat. Med.* **7**, 206–214 (2001).
31. Gicheru, M.M. *et al.* Vervet monkeys vaccinated with killed *Leishmania major* parasites and interleukin-12 develop a type 1 immune response but are not protected against challenge infection. *Infect. Immun.* **69**, 245–251 (2001).
32. Kenney, R.T. *et al.* Protective immunity using recombinant human IL-12 and alum as adjuvants in a primate model of cutaneous leishmaniasis. *J. Immunol.* **163**, 4481–4488 (1999).
33. O'Garra, A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**, 275–283 (1998).
34. Hochrein, H. *et al.* Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J. Exp. Med.* **192**, 823–833 (2000).
35. Brown, M.A. *et al.* B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells. *Cell* **50**, 809–818 (1987).
36. O'Keeffe, M. *et al.* Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood* **101**, 1453–1459 (2003).
37. Hayashi, N. *et al.* Kupffer cells from *Schistosoma mansoni*-infected mice participate in the prompt type 2 differentiation of hepatic T cells in response to worm antigens. *J. Immunol.* **163**, 6702–6711 (1999).
38. Qi, H., Popov, V. & Soong, L. Leishmania amazonensis-dendritic cell interactions *in vitro* and the priming of parasite-specific CD4(+) T cells *in vivo*. *J. Immunol.* **167**, 4534–4542 (2001).
39. Swain, S.L., McKenzie, D.T., Dutton, R.W., Tonkonogy, S.L. & English, M. The role of IL4 and IL5: characterization of a distinct helper T cell subset that makes IL4 and IL5 (Th2) and requires priming before induction of lymphokine secretion. *Immunol. Rev.* **102**, 77–105 (1988).
40. Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23–35 (2003).
41. Ross, T.M., Xu, Y., Bright, R.A. & Robinson, H.L. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. *Nat. Immunol.* **1**, 127–131 (2000).
42. Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C. & Fearon, D.T. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* **271**, 348–350 (1996).
43. Ochsenbein, A.F. & Zinkernagel, R.M. Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* **21**, 624–630 (2000).
44. Mohrs, M. *et al.* Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. *J. Immunol.* **162**, 7302–7308 (1999).
45. Yamaguchi, M. *et al.* Regulation of mouse mast cell surface Fc epsilon RI expression by dexamethasone. *Int. Immunol.* **13**, 843–851 (2001).
46. Murphy, M.L., Cotterell, S.E., Gorak, P.M., Engwerda, C.R. & Kaye, P.M. Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, *Leishmania donovani*. *J. Immunol.* **161**, 4153–4160 (1998).
47. Fleming, T.J., Fleming, M.L. & Malek, T.R. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* **151**, 2399–2408 (1993).
48. Van Rooijen, N. & Sanders, A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* **174**, 83–93 (1994).
49. Ogawa, M. *et al.* Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* **174**, 63–71 (1991).
50. Ato, M., Stager, S., Engwerda, C.R. & Kaye, P.M. Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nat. Immunol.* **3**, 1185–1191 (2002).